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COMMENTARY

Metabolism in the age of 'omes'

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Summary

Much research in comparative physiology is now performed using 'omics' tools and many results are interpreted in terms of the effects of changes in gene expression on energy metabolism. However, 'metabolism' is a complex phenomenon that spans multiple levels of biological organization. In addition rates and directions of flux change dynamically under various physiological circumstances. Within cells, message level cannot be equated with protein level because multiple mechanisms are at play in the 'regulatory hierarchy' from gene to mRNA to enzyme protein. This results in many documented instances wherein change in mRNA levels and change in enzyme levels are unrelated. It is also known from metabolic control analysis that the influence of single steps in pathways on flux is often small. Flux is a system property and its control tends to be distributed among multiple steps. Consequently, change in enzyme levels cannot be equated with change in flux. Approaches developed by Hans Westerhoff and colleagues, called 'hierarchical regulation analysis', allow quantitative determination of the extent to which 'hierarchical regulation', involving change in enzyme level, and 'metabolic regulation', involving the modulation of the activity of preexisting enzyme, regulate flux. We outline these approaches and provide examples to show their applicability to problems of interest to comparative physiologists.

Key words: gene expression, energy metabolism, flux, metabolic regulation, hierarchical regulation.

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Introduction

Recognition of the profound importance of energy metabolism to the lives of animals has led to decades of research on the subject. Once regarded as the province of biochemists, its study has evolved to become broad, integrative, comparative and ecumenical (Suarez, 2011). Much research effort in comparative, ecological and evolutionary physiology (henceforth referred to, collectively, as 'comparative physiology') is directed at estimating rates of energy metabolism (Hochachka and Somero, 2002; Suarez, 2011). Metabolic rates change during transitions between rest and exercise, normoxia and anoxia, fasting and digesting, hibernation and arousal. Climate change concerns have caused a resurgence of research on temperature effects on metabolic rates (Portner and Farrell, 2008). Metabolic rates can change at time scales ranging from seconds to days, weeks or more. Basal, standard, maximal and field metabolic rates vary across species as traits that have evolved over of millions of years (Hoppeler and Weibel, 2005). Comparative physiologists often ask whether changes in rates of energy metabolism that occur during the lives of individuals (or resistance to change despite environmental perturbation) constitute physiological adaptations. Various approaches have been used to address the question of whether variation in metabolic rates across species might represent evolutionary adaptations. Though these problems have been studied for decades, the age of 'omes' has provided the means by which to study the mechanistic links between metabolic flux and profiles of metabolites (metabolome), proteins (proteome), mRNA (transcriptome) and genes (genome). However, 'with great power, there must also come great responsibility' (Lee and Ditko, 1962).

Comparative physiology has had a long and rich tradition of choosing the 'right' animals (Krogh, 1929) as well as choosing appropriate research tools to answer questions. Ours is among the many biological disciplines that greatly benefit from the application of molecular genetic approaches (Cossins and Somero, 2007). These approaches range from quantifying mRNAs coding for one or a few select enzymes or membrane transporters to DNA microarray or proteomic techniques to screen for changes in levels of thousands of mRNAs or proteins. In this commentary, we identify issues in the way observed changes in mRNA or protein are linked to changes in metabolic flux. In addition, we discuss recent studies that offer quantitative approaches that can be used by comparative physiologists to determine how metabolic pathways are regulated to achieve changes in flux.

The problem of hierarchical control

ter Kuile and Westerhoff (ter Kuile and Westerhoff, 2001) propose a paradigm for the analysis of 'hierarchical control' of flux in cells. At the top of the hierarchy is the genome and, going down the hierarchy, this is followed by the transcriptome and the proteome that, of course, represent mRNAs and the proteins they encode. Continuing the use of 'omic' terminology (for better or for worse), at the bottom of the hierarchy is the metabolome [there are those who even refer to a 'fluxome' (e.g. Wittman, 2007)]. The question is whether changes in parameters quantified by the various 'omics' methods result in changes in metabolic flux. Output from one level in the hierarchy (e.g. mRNA levels) is often used to make explicit claims about metabolism, so the first question to address is whether mRNA levels can predict protein levels. In a survey of 21

publications concerning cellular stress responses, Feder and Walser (Feder and Walser, 2005) found that mRNA and protein levels changed stoichiometrically less than half of the time. Other researchers are not so pessimistic. For example, variation in mRNA coding for metabolic enzymes explained >80% of variation in the metabolic rates of *Fundulus* cardiac ventricles *in vitro*, despite large amounts of variation in both mRNA levels and ventricular metabolic rates across individuals (Crawford and Oleksiak, 2007). However, high throughput screening approaches applied to a wide variety of cell types reveals that positive correlation between mRNA and protein levels can range from as low as 9% to as high as 87% (Gracey, 2007; de Sousa Abreu et al., 2009). Most recently, Schwanhausser et al. (Schwanhausser et al., 2011) explored the relationship between mRNA and protein levels encoded by >5000 genes in mouse fibroblasts and found that mRNA level explained only 40% of the variation in protein level. The results of these studies raise a number of issues. First, the nature of such global relationships is influenced by which genes are sampled. In the work of Schwanhausser et al. (Schwanhausser et al., 2011), genes involved in cell signaling and RNA processing frequently showed either unstable mRNA coding for stable proteins or stable mRNA coding for unstable proteins. In contrast, metabolic genes generally displayed high correlation between mRNA and protein stability. Therefore, it is expected that high throughput studies limited to the expression of genes coding for metabolic enzymes would show better correlations between mRNA and protein levels. Second, such studies typically focus on the relationships between mRNA and protein under steady-state conditions; the situation is different when stressors or regulators induce a change to a new steady state (e.g. acclimation). An example from comparative physiology is the work of Buckley et al. (Buckley et al., 2006), which revealed tissue-specific changes in the expression of >200 genes in gobies (*Gillichthys miralibis*) exposed to elevated temperature. Further examination revealed that although a number of mRNAs and their encoded proteins changed in the same direction, the time course of changes differed between mRNA and protein. Third, the technical challenges are such that most studies of animal cells have made use of immortalized cell cultures. Comparative physiologists employing high throughput approaches to explore the molecular underpinnings of physiological responses are faced with many uncertainties about relationships between mRNA and protein. It is useful to consider the various mechanistic factors that may confound the relationships between these.

Lack of stoichiometry in mRNA and protein levels

In the simplest situation, transcriptional regulation determines protein levels such that changes in mRNA synthesis (gene expression) cause changes in mRNA levels (Rabani et al., 2011). The increase in mRNA levels then leads to corresponding changes in rates of synthesis and levels of protein. Thus, in the simplest case, changes in protein level are caused by changes in rates of transcription. When non-stoichiometric changes are seen, the assumption is that the change in protein is not regulated at the level of transcription. In such a framework, there are many possible reasons for false negatives. The first issue is that the denominators for RNA and protein are different. The levels of a specific mRNA are typically measured relative to total RNA, whereas levels of a specific protein are measured relative to total protein. Because total RNA per gram and total protein per gram are independent, they can change in ways that affect apparent stoichiometries in mRNA (relative to total RNA) and protein (relative to total protein). Another factor to consider is the impact of different half-lives for

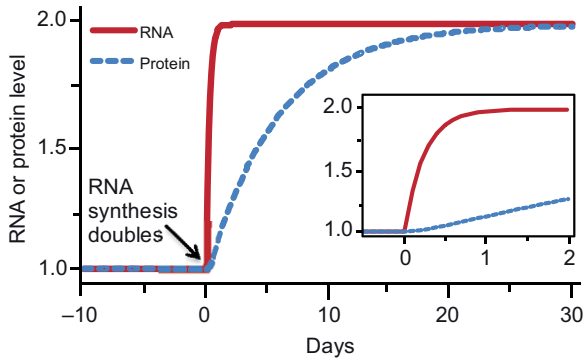

Fig. 1. Changes in RNA and protein levels in response to an instantaneous doubling of mRNA synthesis. The rate of change in RNA level (dR/dt) was calculated from the rate of RNA synthesis (θ) minus the rate of RNA degradation, which is the product of the RNA level (R) and the RNA decay rate ($-m_R$). An RNA half-life of 4 h results in an RNA decay rate (m_R) of 4.1 day^{-1}. The change in protein levels (dP/dt) was calculated from the rate of protein synthesis (the product of RNA level, R, and a constant reflecting translation, ε) minus the rate of protein degradation (the product of the protein level, P, and the protein decay rate, $-m_R$). A protein half-life of 4 days results in a decay rate of 0.17 day^{-1}. The exact values of θ and ε are not critical when expressing values relative to an initial steady-state value of 1 for levels of both RNA and protein. The time course is for an instantaneous doubling of RNA synthesis at day 0, with the inset focusing on changes over the first 2 days.

mRNA and protein. The half-life for protein is typically much longer than that of mRNA, confounding any study that uses fine-scale time courses to assess the mechanistic relationships between mRNA and protein (Fig. 1). With a shorter half-life for mRNA than protein (4 h *versus* 4 days in this simulation), a new steady-state level in mRNA can be reached within 1 day, but almost 3 weeks are needed to see a corresponding change in protein level. In their study on fibroblasts, Schwanhausser et al. (Schwanhausser et al., 2011) found no correlation between mRNA and protein half-lives, making it difficult to design experiments capturing the relationship between these parameters in tissues undergoing remodeling.

Subunits and paralogs

The ability to extrapolate from mRNA to enzyme activity is influenced by the structural complexity of the enzyme. The impact of a change in a single mRNA depends on whether the enzyme is encoded by a single gene (e.g. citrate synthase), paralogs (e.g. lactate dehydrogenase) or multiple subunits, each of which could have paralogs [e.g. cytochrome *c* oxidase (COX)]. In a recent study of temperature-induced changes in cytochrome oxidase activities, the mRNA for very few subunits changed in parallel with observed changes in COX activity. Furthermore, changes in some COX mRNAs were seen under conditions where COX activity did not change (Duggan et al., 2011). Thus measurement of mRNA levels for a subset of subunits for complex enzymes can be uninformative or misleading when applied to control of enzyme levels.

Post-transcriptional regulation of protein synthesis

The synthesis of new mRNA contributes to a pool of mRNA that is sampled by ribosomes to produce proteins. Many post-transcriptional controls affect the general translational machinery, altering global rates of protein synthesis. The efficiency of translation of specific mRNAs can be influenced by gene-specific factors such as microRNA or RNA binding proteins, which may

prevent an mRNA from being translated or degraded. Thus, changes in protein synthesis can occur independent of mRNA levels, and *vice versa*. In their global analysis of the relationships between gene expression and protein levels in mouse fibroblasts, Schwanhausser et al. (Schwanhausser et al., 2011) found that translational regulation is the single most important determinant of protein level.

Regulation of protein degradation

Once proteins are made, their levels can be modified through many mechanisms. Fully functional proteins can be targeted for degradation based on sequences that initiate ubiquitination and degradation in the proteasome. Proteins can be selectively targeted for degradation by individual proteases. For example, mitochondria can use their LON protease to remodel COX, degrading one subunit and replacing it with another. In such a case, protein levels can change without a corresponding change in mRNA.

Post-translational modification of proteins

Many enzymes are subject to regulation by covalent modification. Protein phosphorylation and dephosphorylation are processes central to metabolic regulation, but there are many other types of post-translational modifications that also influence the relationship between enzyme content and catalytic activity.

The processes discussed in this section address the possible mechanisms by which changes in mRNA might not reflect changes in enzyme expression. In the following sections, we address why enzyme concentrations and activities do not always reflect changes in metabolic rate.

What is 'metabolic rate'?

Because many omic studies refer to changes in 'metabolism' or 'metabolic rates', it is beneficial to consider what these mean. At the level of the whole animal, rates of energy metabolism are often measured as rates of O_2 consumption ($\dot{V}_{O_2}$) (Ferrannini, 1988; Lighton, 2008), a method referred to as 'indirect calorimetry' or 'respirometry'. The whole-body $\dot{V}_{O_2}$ represents the sum of mitochondrial respiratory rates in various tissues and organs plus the small contributions made by other O_2-consuming processes (Rolfe and Brown, 1997). In principle, $\dot{V}_{O_2}$ can be used to estimate rates of ATP turnover and, if the nature of the metabolic substrate(s) oxidized is known, flux rates through pathways of substrate oxidation can be calculated (Brand, 2005). However, the use of respirometry for this purpose is problematic in animals that are not in physiological steady state (Ferrannini, 1988) or engage in anaerobic metabolism (Hochachka, 1980), photosynthesis (Rumpho et al., 2000) or chemoautotrophy (Childress and Girguis, 2011). In addition, tissues and organs make up various (often undetermined) fractions of total body mass and account for unknown fractions of whole-body $\dot{V}_{O_2}$. A variable fraction of the mitochondrial respiration rates in tissues and organs may be due to proton leak (Rolfe and Brown, 1997). Thus, deciphering the biochemical meaning of whole-body $\dot{V}_{O_2}$ at the cellular level, e.g. estimating ATP turnover rates and metabolic fluxes, often requires an elaborate combination of approaches. An exception worthy of mention is when animals exercise at or near their maximum aerobic metabolic rates ($\dot{V}_{O_2,max}$). Under these conditions, locomotory muscles account for 90% or more of whole-body $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ (rate of carbon dioxide production) (Taylor, 1987) and rates of mitochondrial proton leak would be expected to be minimal (Brand et al., 1993). In animals that use locomotory muscles of a single fiber type, respirometry can be used to estimate ATP turnover and metabolic fluxes at the cellular level (Welch et al., 2007). The whole animal at rest is therefore a 'mixed bag' of tissues and organs (Wang et al., 2001) in which processes such as membrane transport and biosynthesis dominate as the main energy-consuming processes (Darveau et al., 2002). In contrast, the same animal during exercise at or near $\dot{V}_{O_2,max}$ is physiologically very different, with muscle actomyosin-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase accounting for most of whole-body energy expenditure (Szentesi et al., 2001) (Fig. 2). In insects with asynchronous flight muscles, Ca^{2+}-ATPase rates are considered insignificant in comparison with actomyosin-ATPase activities during flight (Josephson et al., 2000), further simplifying the biochemical interpretation of respirometric data.

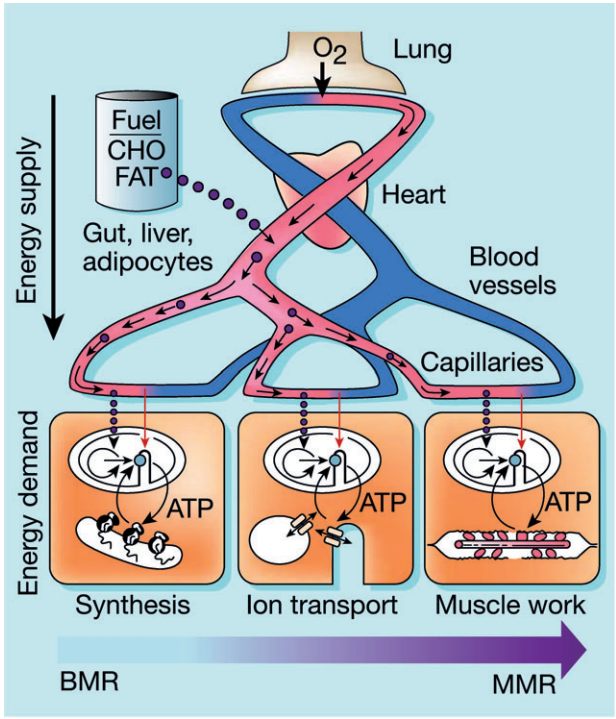

Fig. 2. Multi-level regulation of fluxes related to energy metabolism in air-breathing vertebrates. At basal metabolic rate (BMR), biosynthesis and transport dominate as the main energy-expending processes in various organs. Under these conditions, O_2 and CO_2 fluxes as well as fuel oxidation rates are low. During high-intensity exercise, locomotory muscles account for increasing fractions of whole-body O_2 consumption and CO_2 production rates. At maximal metabolic rates (MMR or $\dot{V}_{O_2,max}$), actomyosin-ATPase is the main ATP-utilizing process and fluxes through fuel oxidation pathways increase to a maximum. Taken from Weibel (Weibel, 2002) with kind permission from the author and publisher.

To further illustrate the complexity of what is loosely referred to as 'metabolism', it is worthwhile to briefly consider the changes that occur during the transition from the fed to the fasted state (Rothman et al., 1991). As the period of fast becomes prolonged, the respiratory quotient, $\dot{V}_{CO_2}/\dot{V}_{O_2}$, declines, indicating that whole-body fuel use switches from reliance upon carbohydrate oxidation in the fed state to fatty acid oxidation in the fasted state. Blood isotope turnover estimates combined with *in vivo* ^{13}C nuclear magnetic resonance spectroscopy reveal that, as hepatic glycogen stores are depleted, gluconeogenesis takes over as the main source of blood glucose. Although the liver uses dietary glucose to synthesize fatty acids in the fed state, during prolonged fasting,

fatty acids are broken down as glucose is synthesized from various precursors. The main point illustrated here is that directions as well as rates of flux change dynamically in response to changing physiological circumstances.

Flux as a system property

The days have long passed when it was simply assumed that enzymes possessing allosteric regulatory properties were 'rate-limiting'. It is now recognized that control of pathway flux is often distributed among many enzymes. Metabolic control analysis (MCA) is now widely used and metabolic biochemists accept, based on the theory underlying MCA as well as empirical results (Fell, 1997), that change in enzyme concentration does not necessarily lead to change in pathway flux. An early example of this comes from the work of Schaaff et al. (Schaaff et al., 1989), which involved the over-expression of eight glycolytic enzymes individually or in pairs in yeast. Despite 3.7- to 13.0-fold increases in enzyme activities, the rate of alcoholic fermentation did not change. Flux is now regarded as a system property rather than the consequence of the rate at which a single 'rate-limiting' enzyme operates. The degree to which individual reactions and transport processes limit flux in pathways is an empirical question. All steps in a pathway could potentially contribute to the limitation of flux and a variety of approaches in MCA have been developed with which to quantify the degree of control exerted by each enzyme (Fell, 1997). The degree to which flux, J, through a pathway is controlled at each step, i, is expressed as a flux control coefficient, C_i. The C_i for any step is the fractional change in pathway flux ($\delta J/J$) that occurs in response to a fractional change in enzyme activity ($\delta e_i/e_i$):

$$C_i = (\delta J/J) / (\delta e_i/e_i) . \quad (1)$$

If a particular step in a pathway has a small flux control coefficient, even a large fractional change in enzyme activity ($\delta e_i/e_i$) can result in a small, perhaps negligible, fractional change in flux ($\delta J/J$) (Fell, 1997). Control analysis has been applied to a wide variety of biological systems and problems in metabolic regulation. For example, applying bottom-up control analysis, Kashiwaya et al. (Kashiwaya et al., 1994) made use of pathway fluxes, maximum enzyme activity (V_{max}) values, Michaelis constants (K_m) for substrates and *in vivo* metabolite concentrations to estimate C_i for many steps in cardiac energy metabolism. This study, like many others, revealed that the control of flux is shared by multiple steps. In addition, the degree of control exerted often changes with physiological state. In the case of the heart, providing insulin and/or ketones causes shifts in C_i among the reactions in glycolysis (Kashiwaya et al., 1994). Top-down MCA (Brown et al., 1990) has been applied to estimate the degree of control exerted by various processes including ATP-hydrolyzing reactions, substrate oxidation and proton leak on mitochondrial respiration (Brand et al., 1993; Buttgereit and Brand, 1995). Conversely, investigation of the degree of control exerted by mitochondrial respiration on various ATP-utilizing processes revealed various degrees of sensitivity to control by ATP supply (Buttgereit and Brand, 1995). The main outcome is perhaps well characterized by stating that 'the cart and the horse' both control each other. Though MCA has been more difficult to apply at the level of the whole animal (Brown, 1994), cardiorespiratory physiologists have devised approaches analogous to MCA to quantify the contribution of various steps to the control of $\dot{V}_{O_2,max}$ (e.g. Jones, 1998). As with metabolic pathways, the flux of O_2 from the external environment to muscle mitochondria is a system property and its control is distributed among the various elements of the 'O_2 transport cascade' (Fig. 2).

Hierarchical regulation analysis

We have pointed out several reasons why changes in mRNA levels do not necessarily lead to changes in enzyme content. Furthermore, changes in enzyme content do not necessarily lead to a change in metabolism or metabolic flux. Whereas the goal of MCA is to identify which steps in a pathway control flux, the additional challenge is to identify the relative importance of changes in enzyme levels (possibly achieved through changes in gene expression) *versus* metabolic regulation (achieved through modulation of enzyme activities) (Bevilacqua et al., 2008).

Hans Westerhoff and colleagues (ter Kuile and Westerhoff, 2001) pioneered 'hierarchical regulation analysis' (HRA) as a quantitative approach to parse out the relative importance of 'hierarchical regulation', involving change in enzyme concentration, [E], and 'metabolic regulation', which involves no change in [E]. It should be noted that hierarchical regulation of [E] does not distinguish between transcriptional, post-transcriptional and translational control. An overview of the origins and derivations of equations is provided in Bevilacqua et al. (Bevilacqua et al., 2008).

The degree to which change in flux J is due to change in [E] at any step i in a pathway can be accounted for by the hierarchical regulation coefficient, ρ_h. On the other hand, the contribution of enzymatic regulatory mechanisms such as mass-action effects and allosteric mechanisms to change in J is measured by the metabolic regulation coefficient, ρ_m. The two are related as:

$$1 = \rho_h + \rho_m . \quad (2)$$

At any enzyme-catalyzed step, ρ_h is a function of the relative change in rate, ∂v_i, divided by the relative change in enzyme concentration, ∂e_i, times the ratio of change in e_i to the change in flux J as expressed in the equation:

$$\rho_h = \frac{\partial \ln v_i}{\partial \ln e_i} \cdot \frac{\mathrm{d} \ln e_i}{\mathrm{d} \ln J} . \quad (3)$$

Assuming that the rate v_i changes in parallel with enzyme concentration e_i, then $\partial \ln v_i / \partial \ln e_i$ equals 1 (ter Kuile and Westerhoff, 2001) and:

$$\rho_h = \frac{\mathrm{d} \ln e_i}{\mathrm{d} \ln J} . \quad (4)$$

Thus, ρ_h represents the ratio of change in enzyme concentration to change in pathway flux. At any step i, the metabolic regulation coefficient ρ_m is the change in an enzyme-catalyzed rate divided by the change in concentration of its substrate, product or allosteric modulator, X, times the ratio of change in X to change in J, such that:

$$\rho_m = \sum_X \frac{\partial \ln v_i}{\partial \ln X} \cdot \frac{\partial \ln X}{\partial \ln J} . \quad (5)$$

Given:

$$V_{max} = e_i k_{cat} , \quad (6)$$

where V_{max} is the maximum enzyme activity measured *in vitro* and k_{cat} is catalytic efficiency [which is generally invariant among orthologous enzymes in animals with similar body temperatures (Hochachka and Somero, 1984)], one way to obtain the hierarchical regulation coefficient, ρ_h, is from the slope of $\ln V_{max}$ plotted against $\ln J$. The metabolic regulation coefficient, ρ_m, is obtained from $1-\rho_h$. This approach allows a quantitative, biochemical dissection of the processes that bring about change in J by distinguishing between 'hierarchical regulation', which results from change in e_i, and 'metabolic regulation', which results from modulation of the activity of pre-existing enzyme. The work

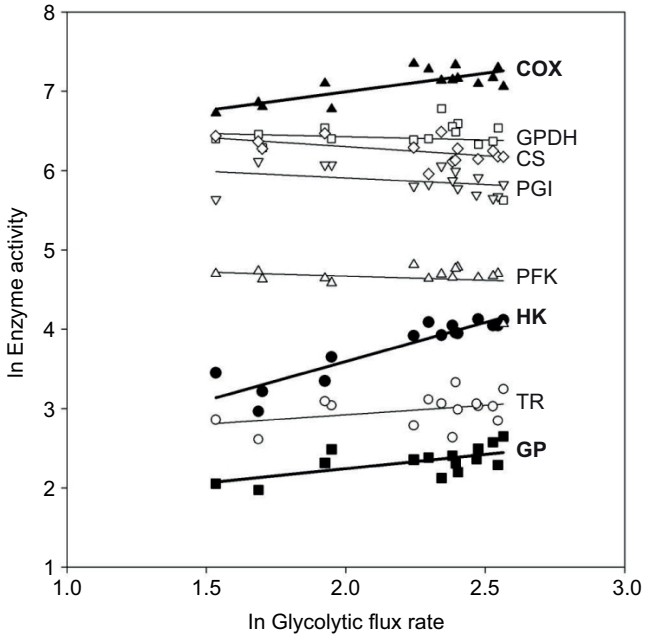

Fig. 3. Relationship between enzyme V_{max} and glycolytic flux (both ln-transformed) at eight reactions in energy metabolism in the flight muscles of 14 Panamanian orchid bee species belonging to four genera. Each point represents data from a single species. Linear regression yields slopes that represent hierarchical regulation coefficients, ρ_h, that are significant for cytochrome *c* oxidase (COX; 0.46), hexokinase (HK; 0.98) and glycogen phosphorylase (GP; 0.36). ρ_h=0 at all other reactions [glycerol 3-phosphate dehydrogenase (GPDH), citrate synthase (CS), phosphoglucoisomerase (PGI), phosphofructokinase (PFK) and trehalase (TR)], indicating that metabolic regulation accounts for interspecific variation in flux at these steps. Phylogenetically independent contrast analysis resulted in support for hierarchical regulation only at HK, providing an explanation for the interspecific variation in flux at this step. Therefore, in addition to morphological and physiological factors resulting in allometric variation in metabolic rates during flight, the allometric variation in metabolic fluxes in muscles during flight is the consequence of both hierarchical and metabolic regulation. Taken from Suarez et al. (Suarez et al., 2005).

of ter Kuile and Westerhoff (ter Kuile and Westerhoff, 2001) on energy metabolism in the parasitic protozoa *Trypanosomabrucei*, *Leishmania donovani* and *Trichomonas vaginalis*, revealed that hierarchical or metabolic regulation may be exclusively responsible for variation in J at certain enzyme-catalyzed steps, whereas at others, both mechanisms may contribute. Variation in J may occur over an evolutionary time frame, as in the case of carbohydrate oxidation rates during hovering flight in Panamanian orchid bees (Suarez et al., 2005). In this work, positive slopes, representing ρ_h values, were obtained at the hexokinase, glycogen phosphorylase and COX reactions (Fig. 3). However, in this application of the method, the variation in J is interspecific and is due to factors including variation in body mass, wing morphology, wing loading and wing beat frequency. The confounding effects of phylogeny and these correlated traits were taken into account by using the method of phylogenetically independent contrasts (Felsenstein, 1985). After 'phylogenetic correction', it was concluded that hierarchical regulation accounts for the variation in J at the hexokinase reaction (metabolic regulation makes no contribution) whereas metabolic regulation accounts for variation in J at all the other reactions examined (hierarchical regulation does not). These examples demonstrate how HRA can be used to address questions concerning physiological as well as evolutionary adaptation in metabolic rates.

Irrespective of whether hierarchical regulation, metabolic regulation or both account for change in flux, the question that next confronts comparative physiologists concerns the proximate mechanisms underlying this change in flux. Metabolite concentrations, of course, do not represent fluxes, and among the attributes of metabolic pathways is that even large, acute changes in flux can be achieved when the concentrations of pathway intermediates change very little (Hochachka, 1994; Fell, 2005). Over an evolutionary time frame, the entire range of interspecific variation in net flux at the phosphoglucoisomerase step in orchid bee flight muscles during hovering can be accounted for by variation in the ratio of [product]/[substrate] of only 0.02. This is most likely too small to be detected empirically (Suarez et al., 2005). The relations among metabolites, enzymes and fluxes are complex (Fell, 2005) and the proper application of metabolomics to questions concerning the control of flux in comparative physiology is well beyond the scope of this article.

As organisms respond or adapt to environmental perturbation, or undergo fluctuations in metabolic rates, the relative contributions of ρ_h and ρ_m might be expected to change over time. In 'time-dependent HRA' (Bruggeman et al., 2006), as applied to yeast metabolism (van Eunen et al., 2009), integration of the regulation between the initial time point t_0 and t is given by:

$$1 = \rho_h(t) + \rho_m(t)\,, \tag{7}$$

where:

$$\rho_h(t) = \frac{\log V_{max}(t) - \log V_{max}(t_0)}{\log v(t) - \log v(t_0)}\,, \tag{8}$$

and v represents transient rates, rather than steady-state J (it does not matter that these authors use log, rather than ln). This allows the relative contributions of hierarchical and metabolic regulation to be tracked over time. Another application concerns the effects of temperature on metabolic rates. HRA was used to quantify the relative contributions of hierarchical and metabolic regulation, as well as direct temperature effects on enzyme activity ρ_T, on temperature-induced (30–38°C) change in metabolic rate in yeast (Postmus et al., 2008). A term to account for temperature effects is added, such that:

$$1 = \rho_h + \rho_m + \rho_T\,, \tag{9}$$

where ρ_T is obtained from:

$$\rho_T = \frac{\Delta \log V_{max,T}}{\Delta \log J}\,, \tag{10}$$

and $\Delta\log V_{max,T}$ is determined by *in vitro* assay at the two relevant temperatures. The remarkable finding in this study is that hierarchical regulation and direct temperature effects both make modest contributions, whereas metabolic regulation is mainly responsible for temperature-induced changes in J in yeast. Application of the 'systems approach' to temperature effects on metabolism by this group has led to a theoretical framework for thermal compensation (Ruoff et al., 2007), a subject of decades-long interest to comparative physiologists (Hochachka and Somero, 2002).

Conclusions

Sydney Brenner, in reference to large-scale data-gathering omics techniques, worries that these might become a substitute for thinking (Brenner, 1999). There are certainly instances in which the data generated by certain omics approaches, particularly transcriptomic

and proteomic, have been used to draw unwarranted or at least questionable conclusions concerning 'metabolism'. It is not unusual to see change in the level of mRNA or protein corresponding to a supposed 'rate-limiting enzyme' or a group of enzymes used to support claims concerning changes in metabolism in the absence of data concerning metabolism. On the other hand, the complex mathematics of 'systems biology', as applied to metabolic biochemistry, often appears inaccessible and inapplicable to problems of concern to comparative physiologists. HRA, in its current state of development, can be brought to bear on many problems in comparative physiology. It provides an accessible theoretical framework and a set of practical empirical approaches that can be adopted to help move the field forward by providing a means by which to mechanistically link change in gene expression with change in metabolic rate.

Glossary

Allosteric
: Referring to regulation of the conformation or activity of a protein, mediated by reversible, concentration-dependent binding of a low-molecular-weight metabolite to a specific site.

Direction of flux
: Used in reference to scenarios involving reversal of carbon flow through linear pathways (e.g. glycolysis *versus* gluconeogenesis) or alteration of rates of flow at branchpoints (e.g. regulation at the pyruvate branchpoint leads to various rates of conversion to lactate, acetylcoenzyme A, alanine, oxaloacetate, etc.).

Flux
: In the simplest situation, the steady-state rate of conversion of an initial pathway substrate to the final end-product, e.g. the rate of glucose conversion to lactate or ethanol.

Fluxome
: All the quantified fluxes of metabolites.

Genome
: All the DNA and the information it encodes in a cell or organism.

LON protease
: ATP-dependent serine protease localized in mitochondria.

Metabolome
: All the low-molecular-weight molecules involved in metabolic transformations catalyzed by enzymes in the proteome.

Omes
: Term coined to describe all biological entities named or renamed using words that end in 'ome'.

Omic tool
: Technique or approach employed to study a specific ome, e.g. DNA microarrays used to study the transcriptome, and proteomic tools used to study the proteome.

Proteasome
: Large protein complex that degrades proteins tagged with ubiquitin.

Proteome
: All proteins translated from the transcriptome.

Transcriptome
: All the messenger RNA transcribed from the genome.

Ubiquitination
: Enzyme-catalyzed reaction that tags proteins with ubiquitin, marking them for degradation.

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